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ON THE DEMONSTRATION OF STAPHYLOCOCCUS-ENTEROTOXIN

[Following is a translation of an article by Dr. Malte Kienitz and Professor Doctor Rudolf Prouner, Director of the Hygiene Institute of the Hanseatic City of Luebeck, in the German-language periodical Zentralblatt fuer Bakteriologie (Central News for Bacteriology), Vol 174, 1958, pages 56-70, submitted on 29 July 1958.]

An attempt was made in a preceding study (28) to indicate the advantages and disadvantages of the biological demonstration procedure for the staphylococcus enterotoxin (Staph.-Enterotox.). Certain methods (e.g., cat test according to Dolman and colleagues), accepted as relatively reliable ones, are suitable for individual examinations also under the conditions obtaining in this country. However, larger test series are, for the most part, precluded owing to considerations of space and funds. The incubated hen egg on the other hand is relatively cheap and can be procured at any time and in the desired quantities. The incubating apparatus requires only a small space; the incubation process itself can be supervised without too much trouble. In view of the reports published by various authors on infection tests with staphylococci on the incubated hen egg (Bibliography, See 30), it was an easy step to conduct a check on the sensitivity of chick embryos to the staph.-enterotox. After a group of tests, we made the statement in a provisional report (29) that the 9-10 day-old chick embryo can -- with 0.15 milliliters of a toxin-containing filtrate -- be injured and caused to die. We will report here the findings of investigations on about 800 incubated eggs.

Material and Methods

1. Staphylococcus Strains

In all, fifty-seven staphylococcus strains were tested: five strains of enterotoxin-forming staphylococci from the Food Research Institute, Chicago (147, 161, 196, 254, S-6); six enteritis strains from the collection of Dr. Williams, London (697, 1074, 2110, 2204, 2341, 2483); four of our own strains that were isolated from children with staphylococcal enterocolitis (127, 165, 20584, 360); and finally fourty-two strains taken

from the material of our Sundries Division. Used as controls were: uninoculated culture media, several strains of micrococcus pyog. var. alb., a branch culture of the Oxford strain and held by the Luebeck Institute. In each case, a test was made of the following properties: coagulase-formation (qualitative, human plasma), Mannitol fermentation, hemolysis, pigment-formation; reaction to six antibiotics in the hole test. A report on the regularly-conducted phage typing (Dr. H. Ph. Poehn, Goettingen) is presented elsewhere. We have tabulated here the hemolysin titers obtained in the tubule test, insofar as it seemed necessary for the elucidation of certain problems.

2. Production of the Filtrates

The problem how to obtain as high-value a toxin filtrate as possible is dealt with in a great number of studies in detail. There is a wide selection of usable culture media, just as there are widely differing nutrients which, upon being consumed, produce symptoms of poisoning, promote the multiplication of enterotoxin-forming staphylococci and the production of staph.-enterotox. A rather recent listing of these is to be found in Ortel (41).

Dack and colleagues (11) showed the presence of staph.-enterotox. in calf's broth culture. Better yields of toxin were achieved with the semi-stiff broth-agar culture medium given by Woolpert and Dack (60), which Burnet (7) also used in a similar mixture for the raising of other staphylococcus toxins. Finally, we would also mention the semi-stiff proteose-peptone-agar culture medium described by Dolman and Wilson (16), and the liquid medium of Favorite and Hammon (17), which consists of 1.5 per cent casein-hydrolysate plus glucose and vitamins. An almost complete survey of the studies concerning this subject is given by Surgalla (51), who, at the same time, reports substantial findings of his own.

One can count on sufficient quantities of staph.-enterotox. only if the inoculated culture medium is incubated (25, 60, 14, 36) in a CO_2 atmosphere (20-30 per cent), which in our experience is very well workable with the usual anaerobic jar in which CO_2 -- controlled by a pressure gauge -- is fed in through an easily installable system of tubes. Following this, one allows such an extent of inflow of air -- or preferably O_2 -- as will leave the top still closing tightly. Incubation temperature is held at +37°C . With the same method, one obtains good yields of other staphylococcus toxins (7, 3, 14, 43) as well. A further factor favoring the production of staph.-enterotox. is the agitation of the cultures (17, 51, 9, 53, 50). Owing to lack of technical possibilities, we have no experiences of our own available. Perhaps the simplest should be the method of Favorite and Hammon (17), who used rotating baby bottles. Surgalla (51) affixed his cultures to a wheel turning at 30-330 revolutions per hour.

The procedures used by us so far can be conducted without too great

expenditure of apparatus. Thus, freshly-isolated staphylococci are inoculated into the special culture media either direct from the blood plate (seven per cent sheep's blood-agar plate) or from a simple grape sugar broth. Strains held for a rather long time were subjected by us -- for activation of the enterotoxin formation -- to a multiple passage on starch-agar according to the directions of Jordan and Burrows (26). Incubation in 30 per-cent CO_2 at+37°C for two to three days. In the six-fold passage recommended, we did intercalate two or three times a sheep's blood-agar plate; however in certain other cases, we worked according to the directions of Knothe (32) with multiple passages of the germs over the incubated hen egg.

The following culture media were used by us.

Medium 1. Peptone broth with 0.1 percent agar, 1 percent starch, pH 6.0, 60 minutes autoclaved at +106°C. Incubation in 30 percent CO_2 at +37°C for five days. We usually used blood-storage bottles that held 450 milliliters of this culture medium or small flasks with a 50 or 100 milliliters content. After conclusion of the incubation the deposits were centrifuged (90 minutes, 4000 revolutions per minute Christ Junior II) and filtered through a Seits-Ek-layer. Since with Seits filtration (60) as with paper filters (49), a portion of the toxins is lost, we now prefer fine-pore membrane filters, such as the No 30 filter of the Goettingen Membrane Filter Company. After running double sterility checks, the filtrates were heated for 30 minutes at +100°C in water and then placed for several hours in a cooling chamber at +4°C; then, they were once more centrifuged (10 minutes, 3,000 revolutions per minute), and stored at +4°C until used. Before the beginning of the test the pH value was tested and the filtrate brought to pH 7.2. At first, we worked exclusively with this culture medium -- described in the directions given by Winkle (58), and used by Moser (38) and also Moser and Mummer (39) in their investigations -- but now use it only for occasional problems.

Medium 2. Mixture same as in Medium 1, but with addition of 3 per-cent glucose, pH 7.2. Decant into 100-milliliter flasks and autoclave at +106°C for 60 minutes. After inoculation with three spots of a plate culture or two or three drops of a liquid culture, incubation under the same conditions as Medium 1, though only for four days in CO_2 atmosphere. For an additional 24 hours, the culture should remain under normal atmospheric conditions at +37°C (60). Then, treatment as for Medium 1.

Medium 3. Mixture as in Medium 1, but addition of 3 per cent glucose and 0.4 per cent agar, pH 7.2. The culture medium is poured into 250-milliliter flasks and autoclaved at +106°C for 60 minutes, then poured off onto Petri dishes (layer thickness - one centimeter). Inoculation with 4-5 drops of a liquid culture. Incubation as for Medium 2. After conclusion of the incubation process, the whole content of the Petri dish is emptied into fine-mesh gauze and carefully squeezed out; the resulting liquid is centrifuged (90 minutes, 5,000 revolutuons per minute), sterile filtered, and treated as with Medium 1 and 2. This culture medium is

particularly suitable for the simultaneous investigation of a larger series of strains.

The filtrates produced in the way described, still contained, for the most part, remains of the α - and β -hemolysins demonstrated in the starting cultures, while the δ -hemolysin was, to a great extent, absorbed during the filtration (56); the dermonecrotic, as well as the lethal toxin were no longer observable (55) after heating at +100°C for 30 minutes. One has to reckon with the disadvantage of reducing the enterotoxin content by 50 percent and more, when one heats the culture filtrates (12, 11). For the obtaining of higher enterotoxin concentrations, numerous precipitation and purification methods (37, 53, 1, 2) -- in addition to some concentration procedures -- are available. The precipitation of the staph.-enterotox. by ammonium sulphate is quite simple (14, 12, 1). Except for a small deviation (longer duration of the dialysis), we followed the directions of Thatcher and colleagues (56) and have been for a time, employing the precipitation of the staph.-enterotox. by the phosphoric acid as well as by the "cold ethanol" methods. Further purification methods can be consulted in Surgalla and colleagues (53, 54), Bergdoll and colleagues (1, 2), as well as Thatcher and colleagues (56). In this matter, at all events, thoroughgoing knowledge of the complicated technique is often requisite, and without it usable findings cannot be achieved (35).

A group of reports exists on the neutralisation of the hemolysins present in enterotoxin-containing filtrates by antitoxic sera. Woolpert and Dack (60) found a neutralising of the hemolysin already with a proportion of 1:10. We work with the staphylococcus serum of the Behring Works (200-fold).

For determining the hemolysins and toxins, we use the following methods: α-hemolysin, titration of the filtrates against a 1 per cent rabbit-erythrocytes suspension in accordance with the directions of Dolman and Kitching (15). β -hemolysin, titration of the filtrates against a 1 per cent sheep-erythrocytes suspension (hot-cold lysis) as in the description given by Surgalla and Hte (52). The dilution stage in both series that still just shows a clear and complete hemolysis is shown as the titer. As a unit serves the so-called MHD (minimum hemolytic dose). This is, according to Dolman and Kitching (15), "the volume of toxin which just suffices to hemolyse completely one cc of a 1% suspension in normal saline of rabbit erythrocytes after one hour incubation at 37°C" and can similarly be used for the β -hemolysin (5, 52).

We normally check the lethal toxins by intravenous or intraperitoneal injection of the filtrates on the white mouse (0.1 milliliter/gram Mouse intraperitoneally, 0.05 milliliter/gram Mouse intravenously). In important tests there is conducted, in addition, the intravenous injection on rabbits (grown animal, 1-1.5 milliliters intravenously). The judgement of the tests was done with the help of the classical studies on lethal toxin (49, 27, 6, 33, 22, 42, 13, 47).

We have here, as in the preceding publications, shown the lethal toxin and the α-hemolysin separately. The long-standing question (34, 57) as to how far those substances are identical and whether the dermonecrotic toxin and the leucocydin should also be included in them, can not be further entered into here. For practical purposes it is convenient to use the separation -- made also by American authors -- whereby we determine, in addition to the hemolysins, only the lethal toxin. Finally, we would remark that even with the most exact execution the applicable prescriptions, one can, with the same strain and the same culture medium, obtain in the end filtrates of differing quality. This experience, common to us also, often forces one to check the filtrates for their hemolysin content before heating, which content stands in certain (admittedly by no means fixed) relationships to the enterotoxin content.

3. Other Data on Methods

The choice and incubation of the hen eggs, injection of the filtrates judgment of the test findings were conducted in the same way as was described in presenting our infection tests (30). From each egg that died, we removed two samples, after a sterile opening, for testing for the presence of staphylococci (grape sugar broth, seven percent sheep's blood-agar plate). Anaerobic culture procedures were not carried out. On growth of staphylococci in one of the two culture media, the egg was eliminated from the valuation if the isolated germs obviously could not originate from the injected filtrates.

The examination of the embryos that had died was limited to the macroscopic observation of the inner organs and the opened head. Histological procedures were not carried out in the tests here being reported.

Results Found

Preliminary Note

The tabulations given to illustrate the problems discussed show, in each case, the whole test. As our effort here was principally to clarify the specificity of the procedure under discussion, it seemed to us appropriate to add explanatory notes to each table.

1. Dependence of the Results Found on the Season

From a comparison of the findings obtained under similar conditions -- in November 1957 and March 1958 (table 1) -- it appears that considerable differences do exist in the sensitivity of eggs laid in the late autumn and in the spring. This fact, known to every chicken breeder, was confirmed through comparative studies made on the effect of cold, through the application of unpurified filtrates, and in infection tests. A further easily-verifiable criterion for the vitality of the eggs resulted from a chance observation. Every one of the eggs examined in autumn with a crack in the shell, caused by handling or transport, as a rule

died after 24 hours; while spring eggs in a similar, deliberately-induced condition, survived several days and, at times, could even be observed to hatch a healthy chick. The small degree of sensitivity of chick embryos to injuries of every sort in late winter and in spring is an important factor, which must be taken into consideration in any repetition of the tests shown here. It is advisable to work in these months with a maximum dose (0.2 milliliters), to be exceeded only after removal of a corresponding quantity of allantoic fluid. Still better is the use of partially purified filtrates (table 3). The seasonally-conditioned sensitivity variations of chick embryos to the staph.-enterotox. and a number of other factors (breed, size of egg, age, and feeding of the hens, etc.) made the testing of the natural mortality rate of uninoculated eggs necessary. This mortality rate (calculated always from the 5th incubation day) -- including chicks that failed to complete hatching -- was between 15 and 17 percent for the months from September to December 1957; for the months January to April 1958, it was between 9 and 10 percent. The last value is in agreement with the findings of the chicken breeder -- who keeps his motor incubator running only at this time (but not in the autumn and early winter) -- who advised us. Working for the first time with eggs of a new strain or even of a new breed, one is well advised to ascertain the natural mortality rate. With regard to the findings presented in Table 1, we have noted also in the succeeding tables the month in which the tests took place.

2. Dependence of the Results Found on the Weight of the Egg

The egg weight plays an important role in the process of investigation. The smaller the egg, the lower the estimate of the dilution factor to which the injected toxin is subject. It must also be noted that large eggs have more white and less yolk than small eggs (10). After concluding at the beginning of our investigations, that the smaller eggs always died first, we used, so far as possible, only the smallest (and cheapest!) eggs as regards both size and weight classes. Comparative vitality tests (infection with micrococcus pyog. var. aur.) on large and small hen eggs showed no significant disadvantage in the small weight class. For extrinsic reasons we chose almost exclusively the eggs of one year old hens of one strain and one breed (brown Italians or white Leghorns). In this way one, in any case, obtains small eggs, since the egg weight increases with the age of the hens (18).

Table 1

Comparative investigations of the sensitivity of 10 day-old chick embryos to the injection of filtrates of enterotoxin-forming staphylococci in November 1957 (I) and March 1958 (II)

Name of strain		Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died hatching
161	I	26	8	9	3	2	1	3
	II	20	2	2	7	1	---	8
196	I	26	4	7	8	3	2	2
	II	20	3	3	4	3	2	5
127	I	16	10	4	1	---	---	1
	II	16	1	5	4	1	1	4
360	I	12	5	2	1	---	1	3
	II	12	2	---	3	3	---	4
20584	I	6	3	2	1	---	---	---
	II	6	1	2	---	1	---	2
2110	I	6	---	2	1	---	1	2
	II	6	---	---	1	2	---	3
1074	I	6	1	1	1	1	1	1
	II	6	---	2	---	---	1	3
2341	I	6	1	1	1	1	---	2
	II	6	1	---	---	1	---	4
697	I	6	1	2	2	1	---	---
	II	6	1	---	---	1	2	2
2204	I	6	---	---	---	---	1	5
	II	6	---	1	---	---	1	4
2483	I	6	2	2	---	1	---	1
	II	6	1	---	1	---	1	3
Oxford	I	20	1	---	---	---	---	19
	II	10	---	---	---	---	1	9
'Empty' broth	I	10	---	1	---	---	---	9
	II	10	---	---	---	---	---	10

Table 2

Tests to obtain the relationships between filtrate dose and death rate on 10 day-old chick embryos (April 1958)

Name of strain	Dose	Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died hatching
	0.05	4	--	1	--	1	--	2
161	0.1	4	1	--	--	--	--	3
(100° C 30')	0.2	4	1	2	--	--	--	1
	0.05	4	--	--	2	--	1	1
196	0.1	4	--	1	--	2	--	1
(100° C 30')	0.2	4	2	--	--	--	1	1
	0.05	4	2	--	--	--	--	2
254	0.1	4	1	--	2	--	--	1
(100° C 30')	0.2	4	--	1	--	1	--	2
	0.05	4	--	1	--	--	1	2
9-6	0.1	4	--	--	--	--	--	
(100° C 30')	0.2	4	1	1	1	--	1	--
	0.05	4	--	--	--	--	1	3
147	0.1	4	--	1	--	--	--	3
(100° C 30')	0.2	4	1	--	1	--	--	2

Table 3

Investigations of the effect of partially purified (precipitation with $(NH_4)_2SO_4$) toxin filtrates without and with addition of Periston N on 10 day-old chick embryos (March 1958).

Filtrate	Dose	Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died hatching
161 K $(NH_4)_2SO_4$	0.1	10	9	1	—	—	—	—
161 K $(NH_4)_2SO_4$	0.1							
Periston N 100 mg/ml	0.1	6	1	—	1	—	—	4
196 K $(NH_4)_2SO_4$	0.1	10	8	1	1	—	—	—
196 K $(NH_4)_2SO_4$	0.1							
Periston N 100 mg/ml	0.1	6	2	—	—	—	—	4
360 K $(NH_4)_2SO_4$	0.1	10	9	—	—	1	—	—
360 K $(NH_4)_2SO_4$	0.1							
Periston N 100 mg/ml	0.1	6	—	1	—	—	3	2
Oxford K $(NH_4)_2SO_4$	0.1	6	—	—	—	—	—	6
Oxford K $(NH_4)_2SO_4$	0.1							
Periston N 100 mg/ml	0.1	6	1	—	—	—	—	5
Periston N 50 mg/ml	0.1	5	—	—	—	—	—	5
Periston N 100 mg/ml	0.1	5	—	—	—	1	—	4
Periston N 200 mg/ml	0.1	5	1	2	—	—	—	2
Periston N 300 mg/ml	0.1	5	1	1	1	—	1	1

3. Dependence of the Results Found on the Age of the Embryos

The clarification of the relationships between the age of the embryos and the sensitivity to the staph.-enterotox. proved to be relatively simple. There is no need to present the findings in tabular form, since the relations were even more consistent than in the case of the infection tests. The 12 day-old chick embryo is only seldom markedly injured, and the 16 day-old chick embryo is on the other hand never markedly injured by 0.2 milliliters of an enterotoxin-containing filtrate. The mortality rates in the control series did not differ from those of the 14-and 16-day old embryos treated with enterotoxin-containing filtrates. Filtrates of stronger enterotoxin-formers sometimes caused 12 day-old embryos to die; but not more than 1/5th of the eggs in the tests were ever affected by this. For our tests, we therefore chose 9-10 day-old embryos.

4. Dependence of the Results Found on the Dose

Fixed relationships between the dose of the injected filtrates and the mortality rates obtained were not perceptible, presumably because of the numbers being still too small in spite of the use of uniform material. An example is shown by Table 2. The chief reason for this is in our opinion, the differing sensitivity -- clearly evident with small quantities of enterotoxin -- of each individual embryo. The correctness of this assumption emerges from the results found in the experiments with filtrate-concentrates, which are reproduced in Table 4. From 400 milliliters of broth (Medium 1) there was extracted, by precipitation with ammonium sulphate, a concentrate of scarcely 50 milliliters, and this was injected in various different dilutions in the egg. But here again the individual reaction of the single egg is still perceivable. Although the dilution corresponded as 1:8 with the starting filtrate, there is no difference from the 1:4 dilution in the action.

Table 4

Example of the effect of various dilutions of a partially purified filtrate (precipitation with $(NH_4)_2SO_4$) of the enterotoxin-forming staphylococcus strain No 161 on 10 day-old chick embryos (April 1958).

Filtrate	Dose	Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died hatching
161 K $(NH_4)_2SO_4$	0,2	6	4	1	-	-	1	-
161 K $(NH_4)_2SO_4$ (1:2)	0,2	4	1	-	3	-	-	-
161 K $(NH_4)_2SO_4$ (1:4)	0,2	4	-	1	2	-	-	1
161 K $(NH_4)_2SO_4$ (1:8)	0,2	4	1	2	-	-	-	1
161 K $(NH_4)_2SO_4$ (1:16)	0,2	4	1	-	-	-	1	2
161 K $(NH_4)_2SO_4$ (1:32)	0,2	4	-	-	-	-	-	4

Table 5

Comparative investigations of the effect of heated and unheated culture filtrates on 9½ days old chick embryos (February 1958)

Name of filtrate	Hemolysin titer α-	Lethal β-toxin		Dose	Number of eggs	Death at hours after injection 24-72	72-96	Hatched or died hatching
161 filtered	256	-	+	0.2	6	2	2	2
161 filtered and heated (100° C 30')	4	-	-	0.2	6	3	1	2
196 filtered	256	128	+	0.2	6	3	2	1
196 filtered and heated (100° C 30')	8	0.4	+	0.2	6	4	—	2
S-6 filtered	64	-	+	0.2	6	2	1	3
S-6 filtered and heated (100° C 30')	0.4	-	-	0.2	6	3	—	3
127 filtered	8	-	+	0.2	6	1	2	3
127 filtered and heated (100° C 30')	0	-	-	0.2	6	2	1	3
165 filtered	256	64	+	0.2	6	—	2	4
165 filtered and heated (100° C 30')	4-8	4	-	0.2	6	—	1	5

Table 6

Investigations of the protective effect of the (Behrings Works) staphylococcus-serum with simultaneous injection of enterotoxin-containing filtrates on 9 days old chick embryos (November 1957)

Name of filtrate	Dose	Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died hatching
161	0.1	6	2	2	1	1	--	--
161	0.1	6	2	1	1	1	--	1
Staph.-Serum	0.1							
196	0.1	6	1	2	1	--	--	2
196	0.1	6	--	2	1	--	1	2
Staph.-Serum	0.1							
360	0.1	6	2	1	--	1	--	2
360	0.1	6	1	--	2	--	--	3
Staph.-Serum	0.1							
Staph.-Serum	0.1	10	--	--	1	--	1	8
'Empty' broth	0.1	10	1	--	--	--	--	9

5. Dependence of the Results Found on the α - and β -Hemolysin Titers of the Filtrates

During the discussion of the infection tests (30), the assumption was advanced that various staphylococcus-hemolysins or -toxins are capable of exerting a pathogenous effect on chick embryos. However, the single dose 0.2 milliliters of a filtrate seems -- as is perceivable from the example of the non-enterotoxin forming strain 165 which produces both α - and β -hemolysin (Table 5) -- hardly to be sufficient. In the heated filtrates, the low α - and β -hemolysin -remains titers may be regarded as insignificant. In spite of the heating, the action of the filtrates of enterotoxin-forming staphylococci on chick embryos remains unchanged and at times it even appears more clearly than in the case of uncooked filtrates. The latter observation has remained incomprehensible to us so far and could only be clarified by means of rather large test series. In order to exclude completely the possibility of an injurious of the hemolysis still contained in cooked filtrates, the filtrates were mixed in

the proportion of 1:1 with the staphylococcus serum of the Behring Works (200-fold), left standing at +37°C for one hour and then injected. The resulting findings in Table 6 show that the staphylococcus serum does not neutralize the substance that is deadly for the chick embryo. On the other hand, polyvenylpyrrolidon (Periston N) possesses a perceptible toxin-removing, "detoxicating," effect. We added this substance, after weighing in a 0.85 per cent NaCl solution, to the filtrates and incubated the mixture at +37°C for one hour. The addition of 100 milligrams Periston N/milliliter could not be exceeded, since the injection of 200 or 300 milligrams Periston/milliliter 0.85 per cent NaCl solution led to the death of part of the embryos. The injections of 100 milligrams Periston N/milliliter 0.85 per cent NaCl solution were well tolerated. The results represented by Table 3 do permit of a provisional statement as to the detoxicating effect of Periston N, but require verification.

6. Control Investigations of 42 Staphylococcus Strains

So far, a check has been made of 42 staphylococcus strains, isolated in cases of wound infections, pyodermians, osteomyelites, etc., and through staff examinations (children's clinic, surgical clinic). We used Medium 3 as the culture medium and tested the filtrate of each strain on six hen eggs. A filtrate was regarded as enterotoxin-containing, if at least three embryos died within 96 hours. Among the 42 staphylococcus strains, we found, according to this criterion, four enterotoxin-formers, three of which belonged to phage group III, and one to phage group II. A detailed report on this section of the tests is made together with Poehn in another place.

Discussion of the Results Found

Only the death of the embryos was used as criterion for the judgement of the injected filtrates. This is a quite rough, but at all events, consistent standard. The observation of the amnio-peristaltis was far less reliable. Other manifestations of life of the embryos were not studied systematically by us. The "natural" deaths of chick embryos appear, as is known, in clusters at the 2-4th and the 18-20th incubation days, and cannot essentially distort the test findings resulting in the period that concerns us from the ninth to the fifteenth day. In the older literature, astonishingly high mortality rates after microsurgical interventions were reported in the first days of the incubation period (Bibliography, see 21). The majority of these studies could not be seen by us in the original. But comparison of the data of Ferret (19), who observed already, after wounding of the shell and shell-skin, 18.75 per cent atrophied ova or defective development (in total 61.54 per cent abnormal ova), with our control series, shows that older chick embryos tolerate small interventions quite well. Knothe (32, 31) expressed the same view. A prerequisite for these results is strictly sterile working, and care that there be optimum incubation conditions. Superfluous mechanical damage is to be avoided. The

capacity for resistance of chick embryos is so great in the period from the 9-15th day, that brief cooling, careful boring through of the shell, piercing of the egg skin with a fine needle, and injection of 0.1-0.2 milliliters liquid in the allantoic cavity present no serious injuries.

The second cause of death in chick embryos under consideration is the infection with pathogenous germs. Apathogenous microorganisms of the most varied sorts are also observable in the uninoubated hen egg (46, 24 and others), and the data on the frequency of such findings show strong variations. Because of the increased danger of contamination, we deliberately abstained from testing the sterility of the egg content before injection of the toxins. We conducted sterility checks only on the eggs only after death. The prepared cultures exhibited growth in 8-12 per cent of all samples. The incidence unsterile eggs was reached when we omitted the washing off of the shell before the beginning of the incubation. Of the isolated germs (aerobic spore-formers, sarcins, yeasts, gram-positive cluster cocci, and others), further examination was made only of the gram-positive cocci. Demonstration of Micrococcus Pyog. var. aur. excluded the valuation of the egg in question. We regarded only so-called "white" staphylococci as apathogenous when there was -- together with negative coagulase reaction -- a lack of Mannit-fermentation and hemolysin production, and where 9-10 day-old chick embryos infected with these germs survived.

We seldom found pseudomonas aeruginosa or B. proteus; even when found, there was naturally some suspicion of contamination during the handling. As compared with the findings presented by Haines (24), the number of unsterile eggs established by us is undoubtedly small.

After the foregoing observations, there should be no doubt that the death of chick embryos after injection of the filtrates of enterotoxin-forming staphylococci is to be attributed to the action of a substance contained in them. In view of the respective pre-treatments of the filtrates, the only thing that can enter into consideration here is the staph.-enterotox. Even if small concentrations of α - or β -hemolysin are present in the filtrates, they are not capable of exercising any pathogenous effect on chick embryos, where a single dose of 0.2 milliliters is applied. The results found and reported here show that an absolutely regular behavior of the chick embryos after injection of enterotoxin-containing filtrates could not be observed. Here lies the most difficult problem of our tests.

The sensitivity -- varying with the seasons of the year -- of the chick embryos to the staph.-enterotox. corresponds somewhat with the S-shaped curve of the egg production of the hen (45). Parallel to the decline of the egg production is to be observed an increase of the embryos that have been late in their development and that are thus more liable to injuries. Knotho (3) has already pointed this out. In the late winter and the spring months we, in any case, were also unable to obtain any

consistent results, though we but seldom found well developed embryos that had died within the first 48 hours after injection. The -- of itself already small -- dose of 0.1-0.2 milliliters filtrate (allantoic liquid about 1.0 milliliters) is further lowered in the allantoic cavity by the unavoidable dilution; and it noticeably injures consequently only that part of the embryos that, in any case, shows little vitality. If the same volume of filtrate contains staph.-enterotox. in a stronger concentration, then the embryos are usually all killed.

The way the staph.-enterotox. acts is still not clear at many points. Apparently it is a matter of a predominantly neurotopic toxin. How the injury of the chick embryos takes place, where it starts, and what conditions are required for this requires further investigation. Understanding of these questions is helped forward by the knowledge of the biogenetic, physical and chemical processes taking place in the incubated hen egg. We would refer here to the literature on the subject (21, 20, 46, 59, 44). The first question one would ask is what conditions -- important for the enterotoxin action -- exist in the incubated hen egg on the 9-10th day. The growth of chick embryos takes place in several periods. The maximum sensitivity to the staph.-enterotox. to be observed at the 9th day coincides with the so-called second growth depression. Simultaneously with this term, the pH values of the yolk and the white of egg are approximately equal (8, 48, 23) and correspond with an average of 7.3-7.4 to that of the blood (8). The same holds true for the amniotic and the allantoic fluid; while the pH value of the embryo body (squeezed juice of embryo) lies around 7.0 (40); individual organs, on the other hand, show a very varying behavior in this respect (8). These findings recall to mind the tests of Berthwick (4), who was only able to determine a reaction to the staph.-enterotox. with a pH of 7.2-7.4 (filtrate, stomach contents of guinea pigs and rabbits). Whether this parallelism is fortuitous remains at present an open question. We have put forward the idea that the enterotoxin injury at the 9-10th incubation day meets an organism that is relatively capable of resisting, which dies or survives according to the degree of its vitality. The decision on this takes place within 72 -- or at the most 96 -- hours after injection. It is possible to think that the pH values that clearly change after the 10th day are of significance for the insensitivity of older embryos to the staph.-enterotox. A consistent late injury of the embryos exposed at the 10th day to the staph.-enterotox. could not be observed. The mortality rates obtained in the test series for the last 2 days before the hatching and during the hatching hardly differ from the corresponding values of the controls. The hatched chicks were given away to persons connected with the institute or the hospital, and grew just like the animals of the same breed who have undergone regular artificial incubation.

Of the many questions still to be answered, let us select just a few. The possible improvement of the results by means of a change of the injection technique, or raising of the injection dose over 0.2 milliliters,

seems particularly important. The same aim could also perhaps be achieved by a pre-injuring of the embryos, through cold for example. Further, the relationships suggested above between the pH value of the filtrates and allantoic fluid, respectively, are still unclarified. Finally, thorough pathological-anatomical examinations of embryos that have died are lacking. It would be technically very difficult but extremely interesting to observe -- under the action of enterotoxin -- embryos loosed from the egg and brought into an appropriate environment.

The practical utility of incubated hen eggs for the demonstration of the staph.-enterotox. is delimited already by the existing statement. In the present form, the procedure brings only qualitative findings, as a result of which one can at any rate speak of strong or weak enterotoxin-formers, having regard to the mortality rates in question. For single examinations, it appears to us that the technical expenditure with this method is too great. In this case, one should avail oneself of one of the biological tests listed in an earlier publication (28). Larger test series, in which the absolutely necessary accompaniment of positive and negative controls present no difficulty, should present the indication for the employment of the chick embryos method. The findings of the suitably conducted cat test according to Dolman and colleagues (Bibliography. See 28.) do, it is ture, make in this case a somewhat better appearance, as Thatcher and Matheson (36) recently were able to show; but then totally different criteria do not, in any case, permit any true comparison. Further, it would be questionable whether one would in this country find the improbable situation of having 200 cats available for test purposes, and whether one could then decide on this, by no means welcome, mass experiment.

The frequency of the appearance of enterotoxin-forming staphylococci has not been sufficiently investigated. Where, out of 42 strains, we found enterotoxin-formers, we do not believe that this is a statistically usable statement. In the list published by Wilson (57) of similar investigations, much higher "percentage rates" are to be found; and here the total number of the strains tested by the authors cited, does not even partially reach the number on which our investigations are based. These differences can be due to the use of differing demonstration procedures. The more sensitive the method, the higher the percentage rate of the enterotoxin-formers discovered. It would be possible to associate oneself with the view of Wilson (57), who states: "... and it is probable that a large percentage of strains produce greater or less quantities of the toxin." It thus remains to be considered, how one is to distinguish clinically insignificant, weak, enterotoxin-formers from the highly toxogenous strains. The quantitative determination of the amount of enterotoxin produced has not been successful with the biological demonstration procedures so far known. If this were at all possible, then it would most likely be by the use of 9-10 days old chick embryos analogously to the successful diptheria-toxin tests of Knothe (31).

Summary

1. The 9-10 days old chick embryo can be injured and caused to die by the injection of 0.1-0.2 milliliters of an enterotoxin-containing culture filtrate in the allantoic cavity. The enterotoxin content of the filtrate pretreated in the usual way (heating at + 100°C/30 minutes) is not high enough to kill all the embryos of a test series.

2. Partially purified and concentrated filtrates contain the toxin in a concentration that is deadly for all chick embryos.

3. By addition of Periston N (Polyvinylpyrrolidon) the toxic action of partially purified filtrates is clearly diminished. The Staphylococcus serum of the Behring Works showed no perceptible neutralization effect in the test with unpurified filtrates.

4. Among 42 staphylococcus strains isolated from clinical infections or examinations of staff there were four enterotoxin-formers, of which three belonged to phage group III and one belonged to phage group II.

5. The demonstration of the staphylococcus enterotoxin on 9-10 days old chick embryos with the use of unpurified culture filtrates is only advisable for large test series. In the present form, the procedure only allows for qualitative statements, while the quantitative determination of the enterotoxin content in culture filtrates is not presently possible.

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